

## Prevalence of equine herpesvirus 2 (EHV-2) in equine ocular disease

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Received July 23, 2019

Accepted April 30, 2020

### Abstract

Equine gammaherpesvirus 2 (EHV-2) has been linked to keratitis and keratoconjunctivitis but has also been isolated in horses showing no signs of disease. The aim of the current study was to assess the importance of EHV-2 infection in the aetiopathogenesis of ocular disease, where the applied treatment failed. Seventy-eight horses with nonhealing ocular disease were examined at the Equine Clinic of the University of Veterinary Medicine and Pharmaceutical Sciences, Brno, Czech Republic, between the years 2009 and 2016. In total, 96 conjunctival swabs were taken and, starting from 2014, peripheral blood leukocytes (PBLs) were also examined in 42 patients. Positive EHV-2 results were detected in 53 ocular swab samples (54.64%) and in 22 PBL samples (51.16%). The horses were divided into three groups according to age, up to 3 years, from 3 to 15 years and older than 15 years. Depending on the clinical presentation, horses were also divided into nonulcerative or ulcerative keratitis, keratouveitis, keratoconjunctivitis, and corneal degeneration groups. The group of young horses had a significantly higher ocular swab positivity compared to the middle group ( $P = 0.01$ ). Increased bilateral ocular occurrence with decreasing age was observed, although it was not significant ( $P = 0.04$ ). Significant correlation was confirmed between PBL samples and ocular swabs ( $P = 0.01$ ). This correlation was even higher in cases of bilateral infection. No significant differences were detected when comparing the groups according to the clinical presentation. This study describes the prevalence of EHV-2 in different age group horses with non-healing keratopathies.

*Veterinary ophthalmology, keratitis, conjunctivitis, viral infection, PCR, horse*

Equine gammaherpesvirus 2 (EHV-2) is a slowly replicating lymphotropic virus which has been associated with immunosuppression, upper respiratory tract disease, general malaise, and poor performance as well as with ophthalmic diseases – keratitis and conjunctivitis (Collinson et al. 1994; Kershaw et al. 2001). It has also been isolated from horses showing no signs of disease. It is suspected that EHV-2 may be associated with reactivation of latent alphaherpesvirus infection (Studdert 1996). Unlike previous studies focusing on the role of alphaherpesviruses, Muscat et al. (2018) suggested that investigation of the gammaherpesviruses EHV-2 and 5, in transport-related disease should not be dismissed, particularly given that transportation may lead to increased shedding, transmission and reactivation of those viruses but not EHV-1 and 4. The role of gammaherpesviruses in equine keratitis and keratoconjunctivitis is still an object of discussion and any specific keratopathogenic mechanism of the virus is still unknown, but latency in ciliary ganglia may be responsible for virus shedding at the ocular surface during stages of reactivation (Brooks et al. 2017). Deletions and insertions in the genome of EHV-2 and EHV-5 have been identified, which can cause differences in virulence and is the reason why horses can become infected by different strains over a lifetime (Franchini et al. 1997; Kershaw et al. 2001; Bell et al. 2006 b). The most common clinical appearance of the disease

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is superficial punctate keratitis. Ulcerative viral keratitis, as a result of epithelial loss subsequent to stromal oedema, is a less common manifestation of the disease. The third form of herpesviral keratitis is macular keratitis. Macular keratitis is rarely seen and it is the result of recurrent episodes of punctate and ulcerative forms. The virus can be detected from conjunctival swabs, corneal scrapings, and biopsies (Hollingsworth et al. 2015). Virus cultivation or virologic-molecular methods can be used for virus detection, with PCR in different modifications being the current method (Fortier et al. 2010). Despite efforts to demonstrate disease causation in horses, the Equine gammaherpesviruses (GHVs) remain a subject of debate regarding their role in clinical disease (Hartley et al. 2013). The purpose of this study was to evaluate the importance of EHV-2 infection in the aetiopathogenesis of nonhealing ophthalmic disease in horses and compare the results of EHV-2 detection in ocular swabs and peripheral blood leukocytes (PBLs) in patients manifesting ophthalmic disease.

### Materials and Methods

#### Case selection

Horses that met the following criteria were included in this study: presented at the Equine Clinic of Veterinary and Pharmaceutical University in Brno, Czech Republic between the years 2009 and 2016, which were treated for ophthalmic disorder by field veterinarians, with a duration of greater than 3 days and with treatment bringing unsatisfactory results.

Details documented from the medical records included the age, breed, unilateral or bilateral ophthalmic disease, duration and nature of the clinical signs, previous and present medication. All the horses included in this study suffered from unilateral or bilateral ophthalmic disease manifested as nonulcerative keratitis, ulcerative keratitis (corneal Fluorescein stain uptake was present), keratouveitis, keratoconjunctivitis or corneal degeneration.

#### Patients

Seventy-eight horses suffering from ophthalmic disease were examined. In addition, the mother of a foal with bilateral keratitis was also examined for diagnostic reasons but was excluded from the study due to the absence of ophthalmic lesions.

Breeds were representative of the hospital populations (Fig. 1). Out of 78 horses, there were 37 mares, 30 geldings, and 11 stallions included in the study. The median age was 9 years, ranging from one month to 29 years. According to age, horses were divided into 3 groups. The first group included young horses from one month to three years old. The second group of middle-aged horses included horses older than three years up to 15 years of age. The last group included horses older than 15 years.

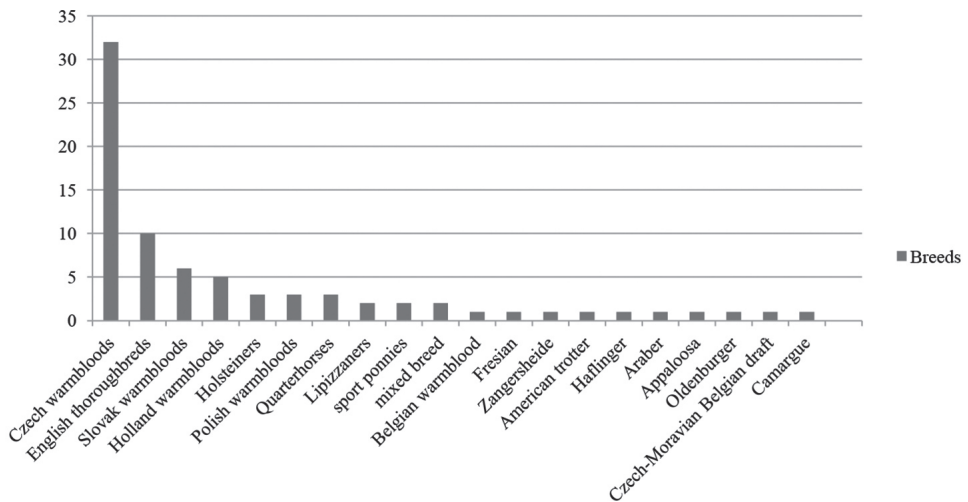


Fig. 1. Horse breeds included in the study

All patients underwent a complete ophthalmic examination. The adnexa, anterior and posterior ocular segments of both eyes were examined with a transilluminator and direct ophthalmoscopy. When uveitis was suspected, tonometry was performed. When posterior segment examination was not possible, transpalpebral ultrasonography was performed with a 10 MHz linear probe. Uncooperative horses underwent the examination under standing sedation with or without topical anaesthesia. Fluorescein staining was performed to assess corneal integrity. As part of the typical diagnostic approach, samples were collected from the corneal lesions and submitted for bacterial culture, fungal culture, and cytologic evaluation. Based on the clinical diagnosis, the eyes were divided into 5 groups – keratoconjunctivitis, keratouveitis, nonulcerative keratitis, ulcerative keratitis, and corneal degeneration group.

Routine ophthalmological examination was followed by taking samples for molecular virological examination. Ninety-six conjunctival swabs and 42 whole blood samples, for detection of the virus circulating in PBLs, were taken for real-time PCR. Each cornea and each ventral conjunctiva was used for a swab sample, using the medial cornea and the conjunctival fornix of the lower eyelid. Topical anesthesia with Benoxi (oxybuprocaini hydrochloridum 4 mg/ml, oph gtt sol 1 × 10 ml, Unimed Pharma spol. s r. o., Slovak Republic) was performed and samples with dry conjunctival/corneal cotton swabs were taken and immediately transported to the laboratory. Each swab was placed into 500 ml sterile phosphate-buffered saline. Desoxyribonucleic acid (DNA) was extracted from 200 ml of suspended material by using the NucleoSpin DNA kit (Macherey-Nagel, Germany). The acquired DNA was stored at -20 °C or immediately used for examination by real-time PCR. Laboratory examination was done with the LC480 polymerase chain reaction (PCR) platform (LightCycler® 480 System, ROCHE, Czech Republic) working at 40 cycles: 95 °C/5 s denaturation, 60 °C/30 s annealing, extension, and data collection. Data were transferred to graphical and numerical forms on the computer display.

A stable part of the viral genome, coding viral enzyme DNA-terminase, was chosen for the detection of EHV-2, and EHV-5 with real-time PCR. Using the PrimerQuest (IDT Integrated DNA Technologies) program, two oligonucleotide primers 19 and 20 base pair (bp) long, were designed from approximately 200 bp long DNA sequence for detection of both viruses. Among them, two oligonucleotide probes were designed, specifically in sequences in which both viruses, EHV 2 and EHV 5 differ, EHV-2 specific marker as Cy5 and EHV-5 specific marker as fluorescein amidites (FAM). Plasmid DNA of known concentration was used for calibration. Signal intensity out of several decimal dilutions of plasmid DNA control was compared to the signal intensity of examined samples.

#### Statistical analysis

Arithmetical mean and median with standard deviation (SD) were used to show the age distribution of horses. Descriptive statistics were used to assess the distribution of the horses in different groups, and the PCR results in the individual groups. Chi-square test was applied to confirm the correlation of PCR results between conjunctival swabs and PBLs. Man-Whitney test was used to confirm the association between eye lesion character and PCR result. ANOVA was used when comparing the PCR results among different age groups.

## Results

### Patients

Seventy-eight horses with altogether 96 eyes, were included in this study, out of which 11 horses suffered from bilateral disease. Out of 96 ocular swabs, 53 tested positive for EHV-2. In 7 cases, there was antiviral treatment with Zovirax (Aciclovirum 5%, ophthalmic gel, Glaxo Wellcome Operations, Great Britain) or Valtrex (Valaciclovirum 500 mg, film-coated tablets, The Wellcome Foundation Ltd. Great Britain) started before referral to the clinic, so the contralateral healthy eye was also swabbed to exclude false-negative results.

### Different age groups

The group of young horses consisted of 10 horses with 15 ocular swabs, i.e. 15.63% from all examined eyes and 12.82% of all horses. The youngest, a one-month-old foal, suffered from bilateral nonulcerative keratitis. The group of middle-age horses consisted of 57 adult horses (67 eyes); 73.08% and 69.79% of all eyes examined, respectively. In the group of older horses, 11 patients were examined and 14 ocular swabs were taken. In the positive contralateral healthy eyes, EHV-2 was also isolated from peripheral blood. The analytical results measured in horses of different age groups are shown in Table 1.

Age groups	Ocular swabs taken (n)	Positive ocular swabs (n and %)	Keratouveitis		Keratoconjunctivitis		Non ulcerative keratitis		Ulcerative keratitis		Corneal degeneration		NBS**	NBS and %
			Bilateral	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral	Bilateral	Unilateral		
			Horses (n)	5	1	3	1	3	1	1	7	4	4	57.14%
			Sampled healthy contralateral eyes	1*	2*				1*					
Young horses	15	86.67%	Positive ocular swabs	4			7		2					
			Horses (n)	1	1	2	3	1	8	1	30	15	15	50%
			Healthy contralateral eyes	5	3		2*		1*					
Middle aged horses	67	52.24%	Positive ocular swabs	6	2	18			9					
			Horses (n)	1	1	2	5	2	1	5	3	60	60	
			Healthy contralateral eyes	1	1		1		1					
Older horses	14	42.86%	Positive ocular swabs		1	2	2		3					

n - number

\* Positive healthy contralateral eyes

\*\*NBS - number of blood samples taken for PCR

### Statistical evaluation of the results

No association was confirmed between the eye lesion character and EHV-2, using Mann-Whitney statistical evaluation at a significance level of  $P = 0.05$ . Significantly higher ocular swab positivity using ANOVA in young horses group compared to middle-aged horses was recorded at  $P = 0.01$ . Less positive ocular swabs were obtained from the group of older horses. A significant relationship between age and positivity of the left eye was documented at a significance level of  $P = 0.04$ , showing a tendency for young horses to have positive left eye conjunctival swabs. However, this could not be confirmed in bilaterally affected eyes ( $P = 0.12$ ) or unilaterally in the right affected eye. A tendency for lower ocular swab with higher age could not be proved at a significant level (Fig. 2). The PCR results from conjunctival samples and PBLs corresponded with each other (90.47%). In 38 out of 42 horses, if there was one or both eyes positive, the peripheral blood samples were positive and vice versa if one or both eyes were negative, no gammaherpesviruses were detected in PBLs. In 21 patients, we obtained positive results from both peripheral blood PCR and conjunctival swabs from one or both eyes. In 17 patients results both from ocular and blood samples were negative. In four patients, we detected EHV-2 in ocular swabs but not in PBLs and in only one patient, ocular samples were negative and gammaherpesvirus was present in PBLs. Using Chi-square test, we found a significant correlation between ocular swabs and peripheral blood samples. In the case of the left eye, the significance level was  $P = 0.01$  (PCR from peripheral blood was positive for 79% of positive left ocular swabs and 35.7% of negative left ocular swabs and for the right eye  $P < 0.001$  (PCR from peripheral blood was positive for 85.7% positive right eye swabs and for 0% of negative right eyes). The relationship between the presence of the virus in PBLs and conjunctival swabs were confirmed at a significance level of  $P < 0.0001$ . When the virus is present in conjunctival swabs from one eye, it will very probably be present in PBLs too. Even higher positive PBLs PCR result probability can be seen in the case of bilaterally positive conjunctival swabs (Fig. 3).

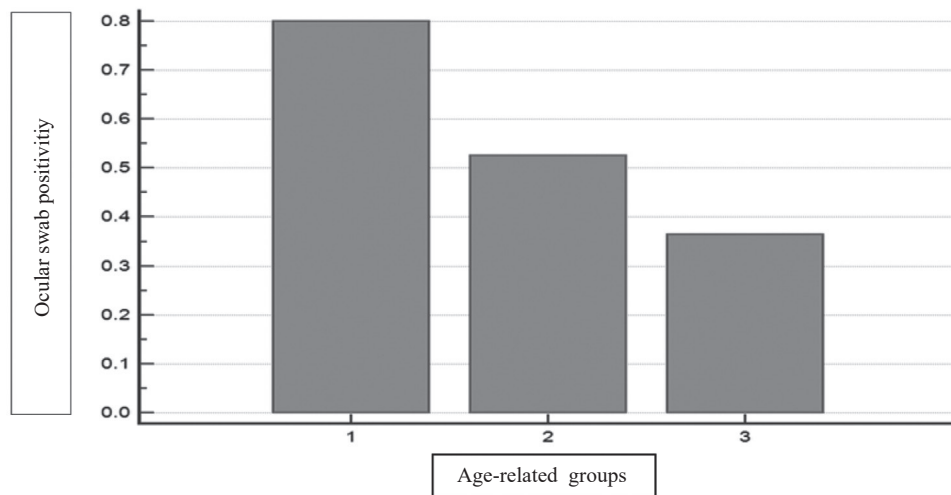


Fig. 2. Comparison of ocular swab positivity in horses according to the age groups using ANOVA (1 - group of young horses, 2 - group of middle age horses, 3 - group of older horses)



for human herpes simplex virus keratitis and the mechanism has been described as the phosphorylation of acyclovir compound by Herpes simplex virus thymidine kinase. The mechanism of the human Epstein-Barr virus and cytomegalovirus and therefore, the animal gammaherpesviruses, is not clear as they do not produce the thymidine kinase enzyme. However, replication of the viruses is significantly impaired (Gnann et al. 1983). In the seventh patient, the EHV-2 virus was only obtained from the eye showing clinical signs of ocular disease.

One conjunctival sample was taken from the horse with a sudden appearance of band corneal opacity. The eye was not painful at the presentation and no infectious agent was confirmed by either cytology, bacteriology or PCR. The lesion did not improve or worsen with therapy or over time. The lesion was diagnosed as corneal degeneration.

Inverse correlation of age and ocular swab positivity was documented ( $P = 0.04$ ), in patients in which the left eye was sampled and in bilaterally affected eyes, but it could not be proven to a significant level ( $P = 0.12$ ). No correlation was recorded in the affected eyes on the right side. This could be attributed to the different sample size of unilateral left or right and bilaterally affected eyes. This inverse correlation has been mentioned by Rushton et al. (2013a) who took samples from 266 Lipizzaners and detected EHV-2 in 160 horses (60.2%). Forty-one horses (15.4%) in peripheral blood samples, 137 horses (51.5%) in nasal swabs and 110 horses (41.4%) in conjunctival swabs were positive using the quantitative PCR (qPCR) molecular method. Follow-up sampling was done after 6, 12 and 18 months and EHV-2 had a significantly higher prevalence in horses younger than 7.5 years, and EHV-5 under 6.9 years (Rushton et al. 2013b). In our study, 86.67% of ocular swabs and 57.14% of PBLs were positive in the young horses group. Rushton et al. (2013 b) suggested that the free housing system in colts and fillies, which brings horses in closer contact and sometimes stress to the horses lower in the herd hierarchy, could be responsible for the increased probability of gammaherpesvirus infection or reactivation. Potential stressors such as restructuring and translocation should be considered to reduce the risk of EHV infections, as environmental stressors may play an important role in EHV reactivation and spread in the equine population (Seeber et al. 2018).

Gammaherpesvirus has been detected in PBLs by several authors (Franchini et al. 1997; Kershaw et al. 2001; Rushton et al. 2013a,b; Rushton et al. 2016). In our study, a significant correlation between the results of ocular and PBLs real-time PCR results ( $P < 0.001$ ) was observed. In the most recent study by Rushton et al. (2016), a similar outcome was documented in Icelandic horses, in which EHV-2 virus was confirmed both in the ocular swabs and PBLs. In our results, this correlation was even higher in horses with bilaterally confirmed ocular infection. Although samples of peripheral blood by themselves are not representative for verifying or excluding the role of gammaherpesviruses in ocular disease, sampling of peripheral blood confirms the presence of gammaherpesvirus in the organism. This raises the question of whether only topical ocular treatment will be sufficient to definitively cure the infection, and whether reoccurrence of the ocular disease is likely.

Detection of equine herpesviruses by molecular virological techniques has been reviewed extensively. As high genomic variability exists in EHV-2 isolates (Bell et al. 2006a), a stable part of the viral genome has to be targeted for PCR detection. In our study, the DNA terminase gene was targeted with uniquely designed oligonucleotide primers. This is only one of many gene regions which can be chosen for gammaherpesviral detection. Other often targeted genes are DNA polymerase (VanDevanter et al. 1996), glycoprotein B (Wong et al. 2010), ORF 64, ORF 74, ORF E1, ORF E6 and IL 10-like gene (Franchini et al. 1997). Glycoprotein H (Nordengrahn et al. 2002), DNA polymerase, DNA terminase, and tyrosine kinase gene were also successfully used for the detection of EHV-2 and EHV-5 (Fortier et al. 2010; Hue et al. 2014). Serology is an essential tool in direct virus detection. However, in clinical practice, this can be more challenging as paired serum

samples taken two weeks apart need to be examined. This would considerably increase the cost of diagnosis.

In our study, ophthalmic ointment Zovirax (aciclovirum 5%, ophthalmic ointment, Glaxo Wellcome Operations) or Virgan (ganciclovir 0.15%, ophthalmic gel, Laboratories Théa) was chosen empirically for controlling viral keratitis. Currently, there is no randomized clinical trial that has investigated the efficacy of acyclovir or ganciclovir in horses with herpesvirus related diseases (Wong et al. 2010). The exact pharmacokinetic action of acyclovir with the EHV has not yet been described. Retrospective studies are suggesting its positive effect in horses affected with EHV-1 (Wilkins et al. 2003; Henninger et al. 2007). Their efficacy in ocular diseases in the horse is unpredictable (Brooks et al. 2017). Although determination of the long-term outcome or statistical evaluation of our therapy was not possible in this study, the patients had a good clinical response and signs of healing after topical acyclovir administration. Evaluation of the therapeutic outcome by both clinical assessment and repeated sampling by PCR tests would be an interesting subject for future studies.

It can be concluded that our study concurs with the findings of Gonzalez-Medina (2015) who claims that GHVs should always be ruled out when treating a horse with keratitis, especially when there is diminished or no response to the initial treatment. Conjunctival swabs and peripheral blood samples are easy to obtain, so PCR testing and treatment with topical antiviral medication could considerably improve the outcome of non-healing corneal lesions in horses, where bacterial or mycotic causes of keratitis are excluded. In the same manner, samples should be taken when an adverse reaction to the corticosteroid treatment applied in presumably non-infectious or immune-mediated disease occurs and not only when the typical clinical presentation of EHV keratitis is observed.

#### Acknowledgement

This project was supported by the Internal Grant Agency of the University of Veterinary and Pharmaceutical Sciences (project No. 97/2014 FVL).

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